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Role of Genomic Imprinting in Wilms' Tumour and Overgrowth Disorders

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Activation of the silent maternal *IGF2* allele has recently been found in approximately half of Wilms' tumour (WTs) examined. This process of imprint relaxation leads to biallelic expression of *IGF2* and it has been suggested that this is a key event in the onset of some WTs. Although it has previously been proposed that the *11p15* chromosome region contains a growth-promoting gene and a tumour suppressor gene, the simplest explanation is that increased expression of the *IGF2* gene is responsible for somatic overgrowth in the BWS and predisposition to tumours. This model explains overgrowth in BWS cases with unbalanced translocations with paternal dup(11p), and cases with balanced maternal translocations which are physically close to the *IGF2* gene. Maternal translocations are envisaged to disrupt the maternal *IGF2* imprint by a mechanism similar to the position-effect variegation mechanism in *Drosophila*. Relaxation of *IGF2* imprinting has also been detected in several patients with the BWS syndrome and a patient with gigantism and Wilms' tumour. Recent gene disruption experiments have shown that inactivation of the mouse *h19* gene leads to biallelic Igf2 expression and extensive proportional overgrowth. This mouse model has parallels with the BWS and WT where it has been found that biallelic *IGF2* expression is accompanied by an epigenetic modification of the *H19* gene. From these data it is possible to speculate that an epigenetic modification of the *H19* gene may be the primary event leading to the relaxation of *IGF2* imprinting.

Key words: Wilms' tumour, imprinted domain, *H19/IGF2*

INTRODUCTION

The first suggestion that Wilms' tumour (WT) had a genetic basis was made in the early 1960s by Miller and collaborators with the observation that WT was associated with aniridia, genitourinary abnormalities, and hemihyertrophy [1]. A decade later, Knudson and Strong proposed a two-hit genetic model of Wilms' tumourigenesis which has since that time provided a key model for investigating the mechanism by which this tumour arises [2]. In 1979, Uta Francke and her colleagues found that patients with the WAGR syndrome had constitutional chromosome 11p13 deletions, which suggested that perhaps this was one of the two genetic events described by Knudson and Strong [3]. Four papers published in 1984 appeared to support this suggestion, in that loss of heterozygosity (LOH) of chromosome 11p alleles occurred in 40% of Wilms' tumours [4–7]. In a review accompanying these papers, the suggestion was made that these data indicated there was a "recessive mutation in (the) aetiology of Wilms' tumour," similar to that found in retinoblastoma [8]. The hypothesised mechanism involved inactivation of a critical gene in 11p13, followed by loss of the normal alleles. This work, together with data from retinoblastoma, provided the first suggestive evidence for what is now termed a tumour suppressor gene.

These findings provided the catalyst to search for the *WT* suppressor gene; however, it was not until 1989 that *WT1* was isolated by positional cloning [9,10]. It is now clear that mutations in *WT1* form only part of the genetic complexity of WTs with only a minority of WTs having detectable mutations. Furthermore, chromosome 11p LOH frequently does not involve *WT1*, with recombination breakpoints occurring distal to this locus, indicating that a second gene, *WT2*, is located in distal 11p [11–13]. In the following discussion it is argued that the *WT2* gene corresponds to the *H19/IGF2* locus.

DISCUSSION

Wilms' Tumour Involves an Imprinted Gene on Chromosome 11

One of the assumptions of the two-hit model is that chromosome 11p LOH should occur randomly. Reeve et al. [7] and Schroeder et al. [14] published data suggesting that maternal 11p alleles were lost preferentially, although no explanation for these data was provided. Subsequently, Wilkins proposed that "transforming genes" (T_r) were present on 11p, and that preferential maternal LOH was due to the differences in the imprinting of the alleles of this gene [15]. A foresighted prediction of this model

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was that the T_r gene would be fully expressed only if the paternal T_r allele was retained, thereby providing an explanation for the selective retention of paternal 11p alleles in Wilms' tumour.

Several other models have been proposed to account for the preferential LOH of maternal alleles in WT. A model proposed by Sapienza and coworkers invoked the existence of a tumour suppressor gene within the context of the two-hit model [16]. In this case the first hit was proposed to involve the inactivation of the paternal allele of a tumour suppressor gene, followed by the loss of the maternal allele. Unlike previous examples of tumour suppressor genes which involve inactivation by mutation, this model invoked that the mutation of the paternal allele was epigenetic due to the activity of an unlinked imprintase. Circumstantial evidence consistent with this model was provided by the observations that in rare Wilms' tumour pedigrees, the disease predisposition locus was not on chromosome 11 [17,18]. These early imprinting models were formulated at a time before the discovery that the *H19/IGF2* locus was imprinted. As discussed below, there is now strong evidence that this locus is involved in the onset of WT.

Insulin-Like Growth Factor II and Wilms' Tumour

WT was the first tumour in which *IGF2* was shown to be transcribed at high levels [19,20], and *IGF2* has now been shown to be transcribed in a number of human tumours [21–26]. Although these early studies suggested that *IGF2* could be involved in tumourigenesis, it was not possible to determine whether *IGF2* expression simply reflected the embryonal nature of the tumour. *IGF2* was, nevertheless, an attractive candidate gene in view of its mitogenic properties and its location at chromosome *11p15*. Further investigations demonstrated that mitotic recombinations (reduction to homozygosity involving gene duplications) on 11p did not include *WT1* but included the *IGF2* locus [11–13]. However, these data were difficult to reconcile in terms of *IGF2* action because the recombination breakpoints were variable and a considerable distance from *IGF2*.

A key discovery was that *IGF2* is imprinted in mice, that is, *IGF2* was transcribed exclusively from the paternal allele in virtually all expressing tissues [27]. Following this finding, the previous data on *IGF2* expression and 11p mitotic recombinations in WT were readily interpreted within the context of a gene dosage model. In this model, 11p LOH and duplication of the active paternal *IGF2* allele were proposed to lead to a double dose of *IGF2* [28]. A double dose of *IGF2* was presumed to lead to increased mitogenesis within the precursor cell population, as one of the first events in Wilms' tumourigenesis. More recently it has been shown that *IGF2* plays a role in preventing apoptosis [29]. It is possible that enhanced levels of *IGF2* within a precursor cell might interfere with the normal balance of apoptosis and differentiation and lead to clonal expansion of a cell population within the developing kidney. The "double dose" and tumour suppressor gene models are clearly different, with one proposing loss of function of a critical gene and the other proposing increased gene transcription.

Relaxation of *IGF2* Imprinting in Wilms' Tumour

The major difficulty with the gene dosage model came from the observation that 11p LOH was found in only approximately 40% of tumours, and therefore the majority of WTs could not be explained by an increase in *IGF2* transcription [4–7]. This paradox led to an analysis of a series of WTs to determine whether the normally silent maternal *IGF2* could be activated in some WTs. Of 19 tumours analysed by reverse transcriptase polymerase chain reaction (RT-PCR) using polymorphisms within exon 9 of *IGF2*, 74% were shown to express *IGF2* from the maternal allele [30,31]. Loss of *IGF2* imprinting (LOI) must therefore occur in a significant proportion of WTs, although the exact contribution of this mechanism will require an analysis of a greater number of tumours. Significantly, not all WTs have *IGF2* LOI or 11p LOH, as some tumours retaining the normal imprinted pattern of *IGF2* expression seen in fetal kidney. An increase in *IGF2* gene dosage or transcription therefore cannot explain the subset of tumours with monoallelic *IGF2* expression. These tumours could represent a subset of tumours which arise by a pathway(s) involving different genetic mutations, or alternatively, they could arise from mutations which affect either the *IGF2* gene or the *IGF2* signal transduction pathway. It will be interesting to determine whether those tumours with normal *IGF2* imprinting patterns have an excess of other genetic alterations, for example, chromosome 1p and 16q LOH.

Chromosome 11p LOH is likely to be an early event in Wilms' tumourigenesis because Southern blot analyses have shown essentially complete loss of the maternal allele, suggesting clonality of the tumour [11–13]. It can be similarly argued that relaxation of *IGF2* imprinting is an early event because of the approximate 1:1 ratio of the expressed paternal and maternal alleles in WTs [30,31]. It is likely that these WTs arise within the fetal kidney from a minor population of cells that express *IGF2* biallelically. In this context, the presence of nephrogenic rests (NRs) in WTs becomes important because they express *IGF2* and have a potential role as precursors of WT [32]. If the relaxation of the maternal *IGF2* imprint occurred stochastically in some cells at a low frequency, then these cells would be randomly distributed throughout the nephrogenic zone, which is the main site of *IGF2* synthesis. This population of cells could then expand as a result of enhanced mitogenesis or reduced apotosis. It is intriguing that approximately 1% of newborn infants have NRs at autopsy [33], raising the possibility that these could arise

from a defect in *IGF2* synthesis such as a relaxation of the maternal *IGF2* imprint. Furthermore, expression from the imprinted maternal allele has been shown to be leaky in mouse tissues [34], suggesting that either a minority population of cells with *IGF2* LOI is present or that there is a low level of expression of the maternal allele in all cells. To date, some NRs have been shown to have mutations in the *WT1* gene, but whether this is a common event has yet to be established [35]. In view of the following: that *IGF2* is expressed in NRs, that *IGF2* stimulates cell division and inhibits apoptosis, and that NRs are detected at high frequency in newborn infants, it seems possible that NRs may arise by an epigenetic change affecting the *IGF2* gene. Whether WTs originate from NRs containing *WT1* mutations, defective *IGF2* imprinting, or both, it is clear that NRs represent an early event and that additional mutations must ultimately be involved in the pathway leading to Wilms tumour.

Although loss of *IGF2* imprinting and duplication of 11p by LOH are consistent with the double dose theory, it might not be immediately apparent how an increase in *IGF2* expression could account for reports showing a lack of *IGF2* expression in proliferating epithelial cells of WTs and tumour xenografts which are predominantly epithelial [36,37]. An explanation for these findings may be provided by the long-held view that WTs involve a disruption of normal kidney development. Since *IGF2* is expressed predominantly in the nephrogenic mesenchyme [38], any cellular event which results in an increase in *IGF2* dosage may lead to a premalignant clonal expansion within the fetal kidney and the formation of NRs as described above. The persistence of these proliferating islands of cells may provide an increased window of opportunity for other genetic events to occur that affect the mitogenesis/differentiation balance. As a consequence of these additional genetic changes, differentiated structures within WTs would be capable of division in the absence of *IGF2*. Generally, epithelial elements within WTs do not express *IGF2*; however, in one study the epithelial structures of some Wilms' tumours were shown to express *IGF2* inappropriately [38]. These findings suggested that at least in some WTs, *IGF2* expression is deregulated and may persist, thereby resulting in the stimulation of cell division or a delay in normal apoptotic mechanisms.

There are two additional potential difficulties with the *IGF2* double dose model: (1) the model predicts that in *IGF2* LOI tumours, the levels of *IGF2* transcripts should be twice that in tumours with normal *IGF2* imprinting. Unfortunately, it is very difficult to measure *IGF2* mRNA accurately at this level of sensitivity. In one report, tumours with *IGF2* LOI were shown to have a twofold but statistically insignificant increase in *IGF2* expression compared to tumours without LOI [39], thus highlighting the considerable technical difficulty in this analysis. In addition, this type of analysis is prone to considerable errors in quantitation because of the extensive histological variation in WTs. As a consequence of histological heterogeneity, the normal method of sampling WT tissue for RNA preparation is inadequate because of the uneven distribution of the *IGF2*-expressing blastemal component. As an indication of this difficulty, in this laboratory, variations in *IGF2* mRNA levels of up to 30-fold have been detected on Northern blots from randomly selected regions of a single tumour (T. Taniguchi, personal communication). The demonstration of a statistically significant twofold increase of *IGF2* mRNA in LOI tumours will require a detailed study using sensitive RNA analytic methods in combination with microdissection techniques to isolate the blastemal regions from LOI and non-LOI Wilms' tumours; (2) The *IGF2* double dose model also requires that *IGF2* protein should be detectable at elevated levels; however, it has been reported that the protein is synthesised at low levels [23]. Although this conclusion could be accurate, the specificity of the antibody was not demonstrated. In this investigation, *IGF2* RNA and protein levels were compared; however, only two WTs were examined, and the numbers of sampling sites and sampling procedures were not described. Furthermore, it was not clear whether the RNA and protein isolates were isolated from the same site. This issue is critically important when examining *IGF2* levels in WTs because of tissue heterogeneity, as discussed above.

Epigenetic Alterations at *H19* and *IGF2* in Wilms' Tumour

The observations that the *H19* gene is physically close to *IGF2* and oppositely imprinted has led to investigations to examine the role of *H19* in Wilms' tumourigenesis [30,37,39,40]. By examining the expression patterns of *IGF2* and *H19*, three classes of Wilms' tumour were identified, having: (1) *IGF2* and *H19* normally imprinted with expression from the paternal and maternal alleles, respectively; (2) maternal 11p LOH such that *H19* expression was very low or absent; and (3) biallelic *IGF2* expression with very low or absent *H19* RNA synthesis.[1]

The finding of a reversal of *H19* and *IGF2* expression on the maternal chromosome is consistent with the enhancer competition model, and led several groups to investigate whether this was associated with methylation changes at the *H19/IGF2* locus. Methylation changes were found to affect the maternally inherited *H19* and *IGF2* genes such that there was a gain of methylation at *H19* and loss of methylation at *IGF2*. These epigenetic changes therefore encompassed the entire *H19/IGF2* imprinted domain, equating gene methylation with gene

[1]Biallelic *H19* and *IGF2* was described in two tumours [30]. However, the levels of *H19* RNA in these tumours was not reported and therefore the biological significance of this finding is unclear.

inactivity. These findings indicated that changes in DNA methylation were either specific for the *H19/IGF2* locus or simply a by-product of a global alteration in methylation levels within the tumour cells. It seemed unlikely that alteration in methylation at *H19/IGF2* in the LOI tumours was a random process because in tumours with normal imprinting patterns, the characteristic methylation patterns of these genes were retained. This group of tumours therefore provided an appropriate control group and led to the conclusion that alterations in methylation in the LOI tumours occurred in a specific manner. The issue of global methylation was examined further by analysing the methylation status of other 11p15 genes in WTs [40]. In this case, it was found that 11p15 genes were hypermethylated regardless of whether WTs had a normal or relaxed *H19/IGF2* imprinting pattern, thereby indicating that methylation changes at *H19/IGF2* were not due to a generalised change in gene methylation. These data collectively demonstrate that a change from a maternal to paternal epigenotype within the *H19/IGF2* imprinted domain is central to the Wilms' tumourigenesis pathway.

Mechanisms of *H19/IGF2* Imprint Relaxation in the Beckwith-Wiedemann Syndrome and Wilms' Tumour

A role for genomic imprinting in tumourigenesis has been established by analysis of the Beckwith-Wiedemann syndrome (BWS; reviewed in reference [41]). The BWS manifests with a predisposition to several embryonal tumours, neonatal hypoglycemia, and overgrowth of several tissues which normally express both *H19* and *IGF2*. Paternal isodisomy has been detected in sporadic BWS patients, suggesting the involvement of an imprinted gene in the vicinity of *H19/IGF2* [42,43]. On the basis of cytogenetic/DNA mapping and linkage studies, it is apparent that the BWS gene(s) is close to or includes the *H19/IGF2* locus. The familial form of the disease may be associated with chromosome 11p abnormalities, suggesting an imprinting mechanism. For example, in patients with balanced translocations, the breakpoints are in two clusters on the maternal chromosome with the majority within 400 kb of *H19/IGF2,* and a minority proximal to HBBC [44]. In families containing balanced 11p15 translocations, the phenotype is only expressed following transmission through females [45,46], suggesting that these translocations interfere with the imprint marking process during oogenesis. Unbalanced translocations are, in contrast, variable over a large chromosomal distance and paternally derived de novo [41]. This heterogeneity of breakpoints is inconsistent with the direct genetic alteration of a specific gene but is compatible with the role of a duplicated paternally expressed gene.

From these data it has been proposed that there are at least two loci on 11p15 which show opposite parental imprinting: the distal locus which contains *IGF2* is paternally expressed, while the proximal locus contains a maternally expressed tumour suppressor gene(s) [44]. A simpler model is that there is only one imprinted locus in 11p15, namely *H19/IGF2,* the imprinting of which is disrupted by a chromatin transmission effect similar to the position effect variegation mechanism found in *Drosophila* [47]. A prediction of this model is that translocation breakpoints may disrupt the normal methylation patterns within the *IGF2* gene. This prediction has been fulfilled in one BWS patient who had maternal translocation breakpoints within the proximal locus (which is the location of the putative tumour suppressor gene), and in whom the *IGF2* gene was shown to be abnormally methylated [44]. The verification of this model will ultimately require demonstration that imprinting of the maternal *H19/IGF2* genes is altered, regardless of the position of the chromosome breakpoint.

The most direct evidence for the role of the imprinted *H19/IGF2* domain in BWS has come from affected individuals with a normal genotype. Weksberg and collaborators found that *IGF2* was biallelically expressed in 4/6 fibroblast cultures from BWS patients [48]. In another investigation, biallelic expression of *IGF2* was identified in the normal kidney, peripheral blood leukocytes, and Wilms' tumour of a child with extensive somatic overgrowth [49]. This child had no features of the BWS except that her growth was proportionate, but her height and weight were well above the 97th percentile. One explanation for the minimal clinical features of this child compared to the BWS, is that the characteristic chromosome 11p duplications/translocations and isodisomy involve large regions of chromosome 11, and therefore genes in addition to *IGF2* could be involved, for example, the hypothetical 11p15 tumour suppressor gene described above [44]. However, when the biallelic *IGF2* expression in BWS fibroblasts is taken into account, this explanation does not seem likely.

The simplest explanation of these findings is that defects in *IGF2* imprinting occur in a mosaic fashion during embryonic development. Consequently, the extent of overgrowth, the type of tissues affected and predisposition to tumour development, would depend on the timing and extent of mosaicism for the defect in *IGF2* imprinting. This mechanism does not necessarily require that the gametic imprint is completely absent, but rather that the imprint is not recognised at the right time during embryogenesis, either because the gametic imprint is flawed, or the imprint recognition mechanism is ineffective. Consequently, if defects in imprint recognition occurred before midline formation this would lead to symmetric overgrowth. In contrast, if inappropriate imprint recognition occurred after midline formation, then asymmetric overgrowth would be observed, and the extent of tissue involvement would depend on which embryonal tissues incurred the defect.

Powerful support for the role of *IGF2* imprinting relaxation in the BWS comes from recent work in the mouse [50]. In this investigation, germline disruption of the maternal *H19* gene led to constitutional biallelic *IGF2* expression and extensive proportional somatic overgrowth. Furthermore, when these mice were crossed with a separate strain containing an inactive *IGF2* paternal allele, the double heterozygote progeny were of normal size [50]. This elegant experiment therefore clearly demonstrated that proportional overgrowth resulted from an inactivation of the maternal *H19* allele, the consequence of which led to an activation of the maternal *IGF2* allele. This experimental system has strong parallels with the situation in Wilms' tumour where biallelic *IGF2* expression is accompanied by an attenuation of *H19* RNA synthesis [37,39,40]. Accordingly, it seems possible that in Wilms' tumour the relaxation of *IGF2* imprinting is preceded by the inactivation of the *H19* gene. In this regard it is intriguing that in the gigantism case described by Ogawa et al. [49], the maternal *H19* allele was found to be abnormally methylated in normal tissues and transcriptionally inactive (T. Taniguchi, personal communication). An epigenetic modification of this nature may therefore be the molecular event that leads to the relaxation of *IGF2* *IGF2* imprinting, tissue overgrowth, and predisposition to Wilms' tumour. Cases such as this, and others with "minimal" BWS features may provide important clues to the role of genomic imprinting in this disease.

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Commentary

Reeve outlines a mechanism that allows him to hypothesize that increased expression of a single gene, *IGF2,* could be responsible for both the somatic overgrowth found in patients with Beckwith-Wiedemann syndrome (BWS) and for the development of Wilms' tumor. This contradicts previous models that have implicated the presence of both a tumor suppressor gene (similar to *WT1*) and a separate growth-promoting gene on chromosome 11p15. It is an attractive construct because of its simplicity, but as Reeve acknowledges, several Wilms' tumors retain normal *IGF2* expression. Clearly, for these tumors, alternative genetic pathways still need to be described.

The mechanism invoked by Reeve postulates "activation" of a maternally imprinted gene. He argues, as have others, (see for example the manuscripts of Moulton et al., in this issue on pages 476–483, and Feinberg on pages 484–489), that the second Wilms' tumor gene (*WT2*) on chromosome 11p corresponds to the *H19/IGF2* locus. Under normal conditions, *IGF2* is expressed from the paternal allele only. Due to (maternal) imprinting the maternal allele is not expressed. Reeve describes a mechanism by which the maternal *IGF2* allele is "activated," a process referred to as imprint relaxation or loss of imprint (LOI). This phenomenon was recently demonstrated in 14/19 Wilms tumors examined. Reeve suggests that loss of *IGF2* imprint represents a key event in the onset of certain Wilms' tumors.